PD-L1 Expression in Non-Small Cell Lung Carcinoma: Comparison Among Cytology, Small Biopsy, and Surgical Resection Specimens

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BACKGROUND: One immunotherapeutic agent for patients with advanced non-small cell lung carcinoma, pembrolizumab, has a companion immunohistochemistry (IHC)-based assay that predicts response by quantifying programmed death-ligand 1 (PD-L1) expression. The current study assessed the feasibility of quantifying PD-L1 expression using cytologic non-small cell lung carcinoma specimens and compared the results with those from small biopsy and surgical resection specimens. **METHODS:** PD-L1 expression was quantified using the IHC-based 22C3 pharmDx assay, with "positivity" defined as staining in ≥50% viable tumor cells; ≥100 tumor cells were required for test adequacy. For cytology specimens, IHC was performed on cell block sections. **RESULTS:** A total of 214 specimens were collected from 188 patients, 206 of which (96%) were found to be adequately cellular, including 36 of 40 cytology (90%) and 69 of 72 small biopsy (96%) specimens. There was no significant difference noted with regard to the feasibility of PD-L1 IHC on small biopsy specimens compared with surgical resection specimens ($P = .99$), or between the percentage of PD-L1-positive cytology and histology (including surgical resection and histologic small biopsy) specimens ($P = .083$). PD-L1 expression was found to be concordant among samples from 21 of 23 patients from whom >1 specimen was collected (91%). There also was no significant difference observed with regard to rates of PD-L1 positivity when comparing age, sex, diagnosis, and specimen site. **CONCLUSIONS:** Quantification of PD-L1 expression is feasible on cytology specimens, and the results are comparable to those obtained from surgical resection and small biopsy specimens, including in matched specimens and using a single predictive IHC marker. Future studies will be necessary to determine the comparative value of other antibodies and their ability to predict response to immunotherapy. ***Cancer Cytopathol* 2017;125:896-907.** *© 2017 American Cancer Society.*

KEY WORDS: endoscopic ultrasound-guided fine-needle aspiration (EBUS-FNA); immunotherapy; programmed cell death protein 1 (PD-1); programmed death-ligand 1 (PD-L1); non-small cell lung cancer (NSCLC); reproducibility; small biopsy.

INTRODUCTION

Lung cancer is the most common cause of cancer-related death worldwide, and non-small cell carcinoma (NSCLC) comprises a majority of cases.[1,2] Although the advent of lung cancer screening programs has increased the overall number of NSCLC cases detected at an early stage,[3] a substantial majority of patients with NSCLC have locally advanced or metastatic disease at the time of diagnosis.[4] In such cases, surgical resection provides little benefit, and tissue diagnosis often is made using a minimally invasive technique. Such techniques include

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transthoracic, computed tomography-guided core needle biopsy and bronchoscopic forceps biopsy with or without electromagnetic navigation, both of which yield a small histologic specimen. Alternately, minimally invasive endobronchial ultrasound-guided (EBUS) fine-needle aspiration biopsy (FNA) or thoracentesis (for cases with concomitant pleural effusion) yield a cytologic specimen. The initial treatment of patients with metastatic NSCLC hinges on the detection of targetable genetic alterations, particularly molecular drivers of lung adenocarcinoma, such as activating mutations in epidermal growth factor receptor (*EGFR*) and rearrangement of the anaplastic lymphoma kinase (*ALK*). Despite the challenge frequently posed by the availability of only limited tissue, clinical tumor genotyping has been validated extensively on cytologic and small histologic specimens.[5–12] Accordingly, joint guidelines from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology,[13] which later were endorsed by the American Society of Clinical Oncology,[14] support *EGFR* and *ALK* testing on cytologic samples, with cell blocks being preferred over smear preparations.

Treatment options traditionally have been limited for patients with NSCLC with locally advanced or metastatic disease without a targetable genetic alteration in the tumor. Recently, blockade of the interaction between programmed cell death protein 1 (PD-1), expressed primarily by T lymphocytes, and its tissue ligand (programmed death-ligand 1[PD-L1]) using monoclonal antibodies has been shown to produce durable clinical responses in patients with advanced NSCLC, including both adenocarcinoma (ADC) and squamous cell carcinoma (SCC).[15–18] Because these immune checkpoint inhibitors may potentially cause severe immune-related adverse events, including pneumonitis and endocrinopathy, biomarkers capable of identifying patients who are likely to respond to therapeutic PD-1 blockade are important for the selection of therapy. In particular, quantification of PD-L1 expression by tumor cells correlates with response to PD-1 blockade with significantly increased response rates in a range of tumors with high levels of expression.[19–21]

Several PD-1-inhibitory monoclonal antibodies currently are available for patients with metastatic NSCLC. Among them, pembrolizumab to our knowledge is the only antibody currently approved by the US Food and Drug Administration (FDA) for first-line therapy, and it is approved only for patients with tumors in which at least 50% of cells express PD-L1. The KEYNOTE-024 trial, on which such approval was based, enrolled patients with tumors in which at least 50% of cells expressed PD-L1 in an immunohistochemistry (IHC)-based companion test (22C3 pharmDx; Dako North America Inc, Carpinteria, California).[22] Pembrolizumab also has received FDA approval as therapy for patients who experience disease progression after platinum-based chemotherapy and those with tumors in which at least 1% of cells express PD-L1,[17] as well as patients with solid tumors of all sites with high microsatellite instability or mismatch repair deficiency that have progressed after prior treatment.[23–25] Although patients in the phase 3 trial of pembrolizumab as first-line therapy had unresectable, stage IV NSCLC, FNA tumor specimens were not permitted for quantification of PD-L1 expression in the clinical trials. Similarly, consensus recommendations from the Papanicolaou Society of Cytopathology[26] and the Pulmonary Pathology Society[27] eschew specific recommendations for the use of cytology preparations in PD-L1 testing. Lack of data and validation may lead clinicians and researchers to doubt the suitability of cytologic and other small biopsy specimens for PD-L1 testing. However, the reality that many patients may only have cytologic or small biopsy specimens available necessitates careful evaluation of the usefulness of these specimens for PD-L1 testing.

Herein, the feasibility and efficacy of PD-L1 quantification using cytologic specimens of NSCLC were explored. First, simple adequacy of the quality and quantity was assessed in a series of cytologic NSCLC specimens. Second, the accuracy of the results was evaluated. Ordinarily, researchers would evaluate interpretations of cytologic specimens by comparing them individually with the results of corresponding surgical resection specimens. In clinical practice, cytologic specimens may represent the only specimens available, especially from patients with advanced disease, thereby hindering such comparison. To address this challenge, 2 analytic methods were used. In the first analysis, bulk results of PD-L1 quantification performed in a series of consecutive cytologic specimens were compared with the results of PD-L1 quantification performed in a consecutive series of histologic specimens (both small biopsy and surgical resection specimens) collected over the same time period. In the second analysis, results of PD-L1 quantification were individually compared among specimens from patients with >1 available specimen.

MATERIALS AND METHODS

After institutional review board approval was obtained, consecutive NSCLC tissue specimens in which PD-L1 expression had been quantified were identified retrospectively in the institutional electronic database archive. Histologic specimens were embedded in paraffin after fixation in 10% neutral buffered formalin (NBF) for at least 4 hours. Cell blocks were prepared for all cytologic specimens as previously described.[28] Briefly, rapid on-site evaluation of biopsy material was performed by a cytopathologist or cytotechnologist for all FNA specimens, and cell blocks were prepared using material fixed in NBF and/or CytoLyt solution (Hologic Inc, Marlborough, Massachusetts), a methanol-based fixative. All thoracentesis and pericardiocentesis specimens were fixed directly in NBF after pellet formation. After centrifugation of the specimen for 5 minutes, the supernatant fluid was removed. Well-formed clots were placed directly in Bio-Wrap (Leica Biosystems Inc, Buffalo Grove, Illinois) and fixed in NBF before paraffin embedding. Alternatively, HistoGel specimen processing gel (Thermo Scientific Richard-Allan Scientific, Waltham, Massachusetts) was added to poorly clotted specimens before solidification at 4 °C. Solidified pellets then were placed in Bio-Wrap and fixed in NBF before paraffin embedding. Both histologic and cytologic specimens were evaluated using the World Health Organization classification for lung tumors[29]; cytologic and other small biopsy specimens were evaluated using the classification proposed by the International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society.[30] IHC analysis of the expression of protein markers including, but not limited to, thyroid transcription factor 1, p40, chromogranin, synaptophysin, and cytokeratin subsets was performed, when necessary, to establish a diagnosis of NSCLC.

PD-L1 expression was quantified in histologic specimens and cytologic cell blocks using the commercially available, IHC-based, 22C3 pharmDx assay according to the manufacturer's instructions. Briefly, all viable tumor cells on an entire slide were evaluated, with the presence of a minimum of 100 viable tumor cells required for the specimen to be considered adequate for quantification of PD-L1 expression. After correlation with a slide stained using hematoxylin and eosin, a pathologist trained in scoring PD-L1 expression scored any perceptible membranous staining ($\geq$1+) of tumor cells and quantified the percentage of viable, PD-L1-expressing tumor cells in the cytology or histology samples. Although multiple pathologists participated in scoring for the study, only 1 pathologist was assigned to each specimen. If an additional sample from a patient became available, its scoring was performed independently and without side-by-side comparison with its antecedent. Staining identified in necrotic cells or pulmonary alveolar macrophages was disregarded. The specimen was considered to be PD-L1 "positive" if $\geq$ 50% of the viable tumor cells expressed PD-L1. All pathologists were trained in scoring PD-L1 expression.

PD-L1 expression was requantified by 2 additional pathologists in a randomly selected subset of cases (10 surgical resection, 10 cytology, and 10 histologic small biopsy specimens) to assess reproducibility. Review pathologists were different from the pathologist who performed the initial quantification. Review pathologists arrived at a consensus without knowledge of the previously reported result. Possible reasons for any discrepancy were noted.

Statistical Analysis

When comparing 2 groups, rates of PD-L1 positivity were compared with the classic Z-test for the difference in percentages, except for rates among specimens from regional versus distant metastatic sites, for which a Fisher exact test was used instead. A Mann-Whitney-Wilcoxon test was performed to compare distributions of PD-L1 expression in different populations. A least squares regression line was computed to compare the distribution of PD-L1 expression by age. The Bonferroni-Holm adjustment was used to correct for multiple comparisons, with adjusted statistical significance defined as $P<.006$ ($P<.050$ before adjustment). All data were analyzed according to the intention-to-treat principle.

RESULTS

A total of 214 lung carcinoma specimens were collected from 188 patients with a median age of 71 years (range, 44-93 years). These included surgical excision/resection specimens of the lung (77 specimens) or other sites (25 specimens). Small histologic biopsy specimens were derived from computed tomography-guided core needle biopsy (39 specimens), endobronchial forceps biopsy (15 specimens), or other histologic biopsy (18 specimens) specimens. Cytology specimens were derived from EBUS-FNA (25 specimens), other FNA (3 specimens), or

TABLE 1. Clinical and Pathologic Details of Patients and Specimens

Characteristic	No.
Specimens	214
Site	
Lung	134
Regional lymph nodes	21
Pleura/pericardium	17
Other thorax/mediastinum	4
Distant metastasis	38
Brain	20
Liver	6
Bone	4
Distant lymph nodes	1
Other	7
Type	
Surgical resection	102
Small biopsy	100
Small histologic biopsy	68
Cytologic biopsy	28
Other	4
Effusion	12
Method	
Histology	174
Cytology	40[a]
Patients	188
Sex	
Male	77
Female	111
Diagnosis	
Adenocarcinoma	141
Squamous cell carcinoma	32
NSCLC, NOS	6
Other carcinoma	9

Abbreviation: NSCLC, NOS, non-small cell lung carcinoma, not otherwise specified.
[a] Twenty-five cytology specimens were collected via endoscopic ultrasound-guided fine-needle aspiration biopsy.

thoracentesis or pericardiocentesis (12 specimens). Detailed clinicopathologic patient and specimen information is outlined in Table 1.

IHC Quantification of PD-L1 Expression

A majority of specimens were adequately cellular for the quantification of PD-L1 expression by IHC, including 36 of 40 cytology specimens (90%), 69 of 72 histologic small biopsy specimens (96%), and 98 of 99 surgical resection specimens (99%). IHC of PD-L1 expression was negative in 150 specimens (70%), positive in 56 specimens (26%), and unsatisfactory for evaluation in 8 specimens (4%). In 38% of adequately cellular specimens, $\geq$15% cells expressed PD-L1. Rates of PD-L1 positivity stratified by specimen type and diagnosis are shown in Table 2. Cytologic specimens were more likely to be PD-L1 positive compared with histologic specimens (39% vs 25%; $P = .083$), particularly small histologic biopsy specimens (39% vs 23%; $P = .094$). However, these associations did not reach statistical significance. There also was no significant difference in the rates of PD-L1 positivity observed among 5 other variables (Table 3).

Because the vast majority of cytology specimens (38 of 40 specimens) were fixed directly in NBF, the effect of CytoLyt fixation on the technical performance of PD-L1 quantification could not be fully assessed. The first cell block derived from material fixed in CytoLyt was an EBUS-FNA of a primary lung ADC in a 69-year-old man. The second was an EBUS-FNA of a primary lung SCC in a 73-year-old man. Both specimens were found to be negative for PD-L1 expression; neither had a correlating histologic specimen.

Matched Specimens

A total of 23 patients had ≥ 2 specimens collected, and PD-L1 expression was concordant among paired or triplicate samples from 21 of these patients (91%). Comparison of cytology and small biopsy specimens versus surgical resection specimens are presented in Table 4; all other comparisons of PD-L1 expression in patients from whom $\geq$2 specimens were collected are shown in Table 5. Representative images from 2 of those patients are presented in Figures 1 and 2. The first discordant case pair consisted of resections of 1 of 2 primary tumors and, 2.5 years later, after adjuvant chemotherapy and pulmonary and cerebral external beam radiotherapy (EBRT), a cerebral metastasis of a lung ADC from a 60-year-old man. IHC demonstrated that approximately 50% of tumor cells ("positive") in the primary tumor expressed PD-L1, whereas approximately 20% of tumor cells ("negative") in the brain metastasis expressed PD-L1. The second discordant case pair consisted of EBUS-FNA and thoracentesis specimens of a primary lung ADC and, 5 months later, *MET*-overexpressed lung ADC in a pleural effusion from an 82-year-old woman after cerebral EBRT, only after she presented with widely metastatic disease. IHC demonstrated that approximately 10% of tumor cells ("negative") in the primary tumor expressed PD-L1, whereas approximately 80% of tumor cells ("positive") in the pleural effusion expressed PD-L1.

Reproducibility

PD-L1 expression was requantified in a subset of cases (10 surgical resection, 10 cytology, and 10 histologic small biopsy specimens) to assess reproducibility, including 4

TABLE 2. PD-L1 Immunohistochemistry Stratified by Specimen Type

Specimen Type (No.)	% Adequate (No.)	% Inadequate (No.)	% PD-L1 Positive (No.)[a]	Diagnosis		
				% Adenocarcinoma (No.)	% Squamous Cell Carcinoma (No.)	% Other Carcinoma (No.)
Cytology (40)	90 (36)	10 (4)	35 (14)	70 (28)	15 (6)	15 (6)
Other small biopsy (72[b])	96 (69)	4 (3)	22 (16)	72 (52)	21 (15)	7 (5)
Resection (102)	99 (101)	1 (1)	26 (26)	77 (79)	14 (14)	9 (9)
Total (214)	96 (206)	4 (8)	26 (56)	74 (159)	16 (35)	9 (20)

Abbreviation: PD-L1, programmed death-ligand 1.
[a] Positive was defined as staining in >50% of tumor cells.
[b] Histologic small biopsy specimens included computed tomography-guided core needle biopsy (39 specimens), endobronchial forceps biopsy (15 specimens), and other core needle or endoscopic biopsy (18 specimens).

TABLE 3. Comparison of PD-L1 Positivity Rates for 214 Total Specimens

Characteristic	PD-L1 Positive, %	*P*	90% Power[a]
Sex			
Female	33	.015	172
Male	18		
Method			
Cytology	39	.206	262
Surgical resection	26		
Other small biopsy	23		
Specimen type			
Small biopsy	25.8	.99	4,100,000
Surgical resection	25.7		
Diagnosis			
Adenocarcinoma	28	.202	328
Squamous cell carcinoma	18		
Age, y			
<71	30	.347	1235
≥71	24		
Site			
Regional	37	.138	321
Distant metastasis	25		

Abbreviation: PD-L1, programmed death-ligand 1.
[a] Number of specimens of each type that would be required to obtain a power of 90%.

cases with PD-L1 expression within approximately 10% of the 50% cutoff value for positivity. An interpathologist quantification discrepancy of 10% to 20% (median, 15%) was present in 5 of 30 cases, including 3 cytology cases (2 thoracentesis specimens containing ADC and 1 pericardiocentesis specimen containing NSCLC, not otherwise specified), 1 computed tomography-guided core needle biopsy specimen of an ADC, and 1 ADC surgical resection specimen. The final interpretation of positive versus negative did not change in any case.

DISCUSSION

Quantification of PD-L1 expression plays a key role in assessing the likelihood of response to PD-1 and PD-L1 inhibitors among patients with advanced NSCLC. Such quantification also has a potential role in predicting patient response to PD-1 inhibitors in other tumor types, as well as in novel anti-PD-L1 and immunotherapeutic combination therapies.[19–21,31,32] Because patients are subject to rare, but potentially severe, immune-related adverse events, it is prudent to attempt to predict the likelihood of response before initiating such therapy.[33] The clinical trial on which the FDA based its approval of pembrolizumab (PD-1 inhibitor) as first-line therapy for patients with advanced NSCLC did not allow for the enrollment of patients with FNA or effusion cytology tumor specimens only,[22] possibly leading clinicians to doubt the usefulness of such specimens for PD-L1 testing. Unfortunately, patients with NSCLC often present with advanced disease. Minimally invasive techniques, including cytologic biopsy, are increasingly the diagnostic method of choice and sole available tissue specimen in such cases.[34,35]

Feasibility of IHC Quantification of PD-L1 Expression Using Cytologic Specimens

The results of the current study demonstrate that cytologic specimens of NSCLC provide sufficient cellularity for the quantification of PD-L1 expression in a majority of cases. To the best of our knowledge, previously reported data regarding the feasibility of quantification of PD-L1 expression are limited. In their study comparing PD-L1 expression between 97 cytologic EBUS-FNA specimens of primary lung malignancy, at least 70 of which were NSCLC and a subset for which concomitant histologic transbronchial biopsy specimens were available, Sakakibara et al noted that only 1 cytologic cell block contained <100 tumor cells.[36] Furthermore, FNA specimens had a significantly greater number of tumor cells and less crush artifact

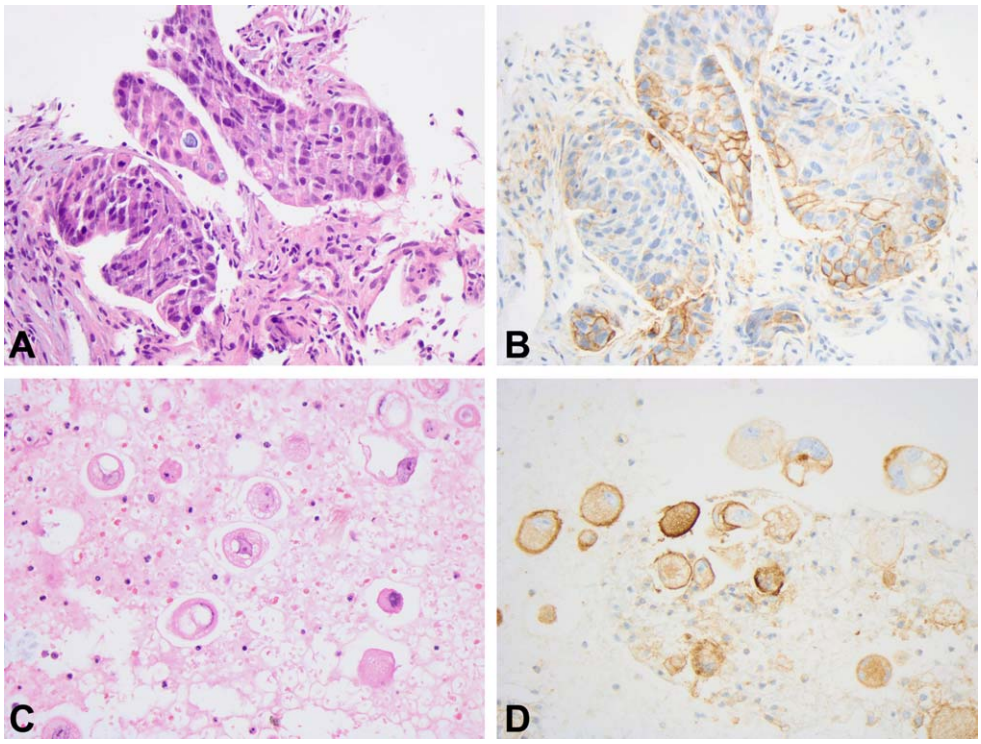

Figure 1. Primary lung adenocarcinoma specimens collected from a male smoker aged 60 years. (A) Endoscopic forceps biopsy of an endobronchial mass (formalin-fixed, paraffin-embedded tissue, H & E; original magnification × 400). (B) Immunohistochemical analysis of programmed death-ligand 1 (PD-L1) expression demonstrated ≥1+ membranous staining in approximately 80% of tumor cells ("PD-L1 positive"). (C) Pleural fluid was collected 11 days later (cell block, H & E; original magnification × 400). (D) Immunohistochemical analysis of PD-L1 expression demonstrated ≥1+ membranous staining in approximately 75% of tumor cells ("PD-L1 positive").

compared with the tissue biopsies. However, that study included 27 neuroendocrine carcinoma specimens (28%), the small cell variant of which may demonstrate high cellularity and extensive crush artifact.[36] In the current study, approximately 90% of cytologic specimens, all of which were NSCLC, provided sufficient cellularity for the quantification of PD-L1 expression.

The current study also indicates that the results of PD-L1 expression testing are comparable among surgical resection, cytology, and other small biopsy specimens of NSCLC. The percentage of specimens in which ≥50% of viable tumors cells expressed PD-L1 (27%) was similar to that noted in the initial phase 1 clinical trial that formally validated PD-L1 expression in NSCLC as a biomarker of clinical response to PD-1 inhibition (23%).[37] There was no significant difference in PD-L1 expression noted between small biopsy (25.8%) and surgical resection (25.7%) specimens, and PD-L1 expression was found to be higher in cytologic (39%) compared with histologic (25%) specimens. The results of the current study suggest that quantification of PD-L1 expression using cytologic specimens is likely to capture patients who will respond to therapy.

Matched Specimens: Small Histologic Biopsy and Surgical Resection

To the best of our knowledge, only a few previous studies have compared the results of PD-L1 testing in NSCLC surgical resection and matched biopsy specimens. In a matched study comprising 160 patients, Ilie et al noted disparate PD-L1 expression in surgical resection and matched small biopsy specimens.[38] The rate of discordance was 48% when both tumor and immune cells were evaluated and 19% when only the former were evaluated. Potential reasons to account for the difference between the results of Ilie et al[38] and those in the current study include the size of the cohort of matched samples and tumor heterogeneity. Furthermore, the anti-PD-L1 antibody used by Ilie et al (SP142) scores both tumor and immune cells.[38] In addition, it was demonstrated to have significantly lower

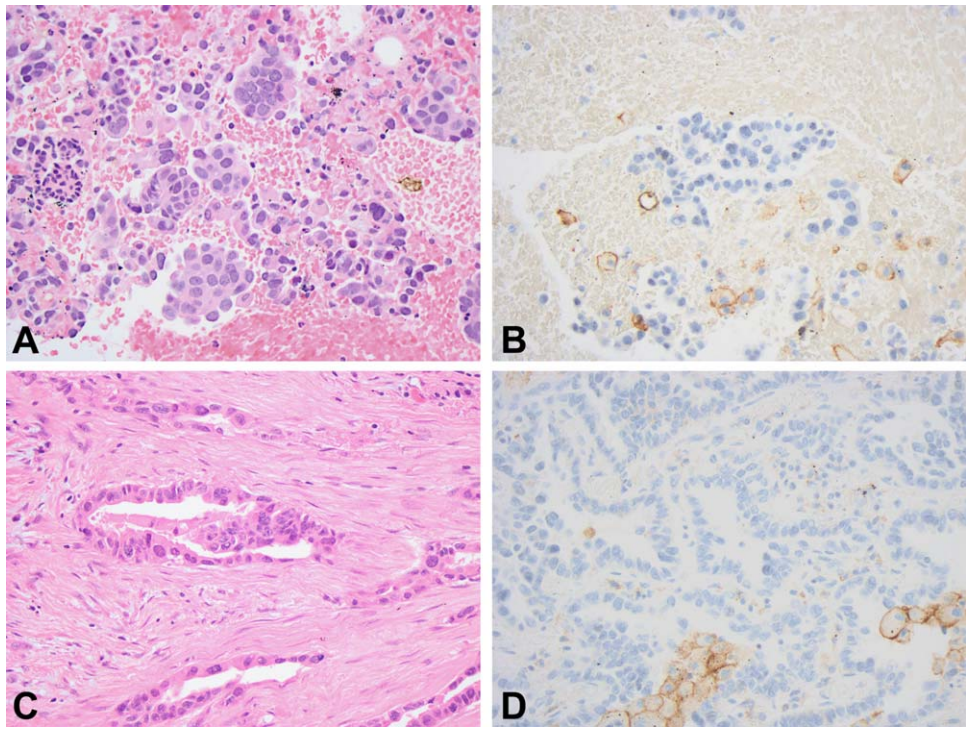

Figure 2. Primary lung adenocarcinoma specimens collected from a female smoker of East Asian ancestry aged 64 years. (A) Endobronchial ultrasound-guided fine-needle aspiration biopsy of a lung mass (cell block, H & E; original magnification × 400). (B) Immunohistochemical analysis of programmed death-ligand 1 (PD-L1) expression demonstrated ≥1+ membranous staining in approximately 10% of tumor cells ("PD-L1 negative"). Staining of pulmonary alveolar macrophages was disregarded. (C) Lobectomy was performed 11 weeks later (formalin-fixed, paraffin-embedded tissue, H & E; original magnification × 400). (D) Immunohistochemical analysis of PD-L1 expression demonstrated ≥1+ membranous staining in approximately 10% of tumor cells ("PD-L1 negative").

analytical sensitivity compared with the 22C3 pharmDx assay in a comparison of 4 different PD-L1 IHC assays collaboratively effected by pharmaceutical, diagnostic, and academic organizations ("Blueprint" comparison).[39,40]

Matched Specimens: Cytology and Histology

In contrast, Kitazono et al observed a concordance rate of 92.4% when comparing PD-L1 expression at a lower "hybrid score" (derived from a combination of staining intensity and distribution) in 79 surgical resection and matched small biopsy specimens of NSCLC.[41] It is interesting to note that 1 of 12 transbronchial needle aspirations demonstrated PD-L1 expression discordant from its matched surgical resection specimen. Using a higher "hybrid score," concordance decreased (83.5%), an observation the authors attributed to tumor heterogeneity.[41] Unfortunately, the anti-PD-L1 antibody used by Kitazono et al (4059; ProSci Inc, Poway, California) is neither monoclonal nor among those used in conjunction with therapies that currently are approved by the FDA or in clinical trials as outlined in the Blueprint comparison. Furthermore, although all viable tumor cells on an entire slide were evaluated in the current study, Kitazono et al counted only 5 random and nonoverlapping fields per slide.[41] This difference in scoring may explain why even at a positivity threshold of ≥50% of cells, there was concordance among all samples originating from the same site in the current study (Tables 4 and 5).

Beyond the 12 transbronchial needle aspirations reported by Kitazono et al, to our knowledge there are only limited data comparing PD-L1 expression in matched cytology and histology specimens. Skov et al reported an overall agreement of 85% at a cutoff value for positivity of 1% expression and 94% overall agreement at a 50% expression cutoff value when using the 22C3 antibody to evaluate 86 cytology-histology lung malignancy pairs, as many as 77 of which were NSCLC. They obtained similar results when using the 28-8 antibody (pharmDx; Dako

TABLE 4. Results of PD-L1 Quantification by IHC in Paired Samples: Cytology and Small Biopsy

	Specimen 1				Specimen 2		
Patient	Type	Diagnosis	PD-L1 Expression, %	Time Since Last Specimen Collection	Type	Diagnosis	PD-L1 Expression, %
Cytology vs cytology							
1	EBUS-FNA	ADC	Unsatisfactory	6 d	Pericardiocentesis	NSCLC, NOS	Negative (20)
2	EBUS-FNA	ADC	Positive (90)	86 d	Thoracentesis	ADC	Positive (80)
3[a]	EBUS-FNA	ADC	Negative (10)	5 mo	Thoracentesis	ADC	Positive (80)
Cytology vs histology							
4	Thoracentesis	ADC	Positive (75)	9 d	Bronchoscopic Bx	ADC	Positive (80)
5	EBUS-FBA	ADC	Negative (10)	4 mo	Surgical resection	ADC	Negative (10)
6	CTG-CN Bx	ADC	Unsatisfactory	17 d	EBUS-FNA	ADC	Negative (5)
7	EBUS-FNA	NSCLC, NOS	Positive (>90)	34 d	Surgical resection	LCNEC	Positive (>90)
8	EBUS-FNA	SCC	Negative (0)	33 d	Surgical resection	SCC	Negative (1-4)
Small histologic Bx vs surgical resection							
9[b]	CTG-CN Bx	SCC	Negative (0)	57 d	Surgical resection	SCC	Negative (40)
10	Surgical resection	ADC	Negative (20)	14 mo	CTG-CN Bx	ADC	Negative (20)
11	CTG-CN Bx	ADC	Negative (1-4)	28 d	Surgical resection	ADC	Negative (0)

Abbreviations: ADC, adenocarcinoma; Bx, biopsy; CTG-CN, computed tomography-guided core needle biopsy; EBUS-FNA, endobronchial ultrasound-guided fine-needle aspiration; IHC, immunohistochemistry; LCNEC, large cell neuroendocrine carcinoma; NSCLC, NOS, non-small cell lung carcinoma, not otherwise specified; PD-L1, programmed death-ligand 1; SCC, squamous cell carcinoma.
[a] Discordant case.
[b] A third specimen (surgical resection 25 days later) with a diagnosis of ADC had PD-L1 expression of 0%.

TABLE 5. Results of PD-L1 Quantification by IHC in Paired Samples: Histology

	Specimen 1				Specimen 2		
Patient	Type	Diagnosis	PD-L1 Expression, %	Time Since Last Specimen Collection	Type	Diagnosis	PD-L1 Expression, %
Small histologic Bx vs small histologic Bx							
1	Endoscopic Bx	ADC	Negative (0)	53 d	Other	ADC	Negative (0)
2	CTG-CN Bx	ADC	Negative (0)	Concurrent	CTG-CN Bx	ADC	Negative (0)
Surgical resection vs surgical resection							
3[a]	Surgical resection	ADC	Negative (0)	Concurrent	Surgical resection	ADC	Negative (0)
4[b]	Surgical resection	ADC	Positive (50)	2.5 y	Surgical resection	ADC	Negative (20)
5	Surgical resection	ADC	Negative (0)	Concurrent	Surgical resection	ADC	Negative (0)
6[a]	Surgical resection	ADC	Negative (0)	Concurrent	Surgical resection	ADC	Negative (0)
7	Surgical resection	ADC	Positive (70)	7 mo	Surgical resection	ADC	Positive (90)
8	Surgical resection	ADC	Negative (0)	50 d	Surgical resection	ADC	Negative (0)
9	Surgical resection	ADC	Unsatisfactory	Concurrent	Surgical resection	ADC	Negative (10)
10	Surgical resection	ADC	Negative (0)	Concurrent	Surgical resection	ADC	Negative (0)
11	Surgical resection	ADC	Negative (0)	Concurrent	Surgical resection	ADC	Negative (15)
12	Surgical resection	ADC	Positive (60)	7 mo	Surgical resection	NSCLC, NOS	Positive (50)

Abbreviations: ADC, adenocarcinoma; Bx, biopsy; CTG-CN, computed tomography-guided core needle biopsy; IHC, immunohistochemistry; NSCLC, NOS, non-small cell lung carcinoma, not otherwise specified; PD-L1, programmed death-ligand 1.
[a] A third specimen (concurrent resection) with diagnosis of ADC had PD-L1 expression of 0%.
[b] Discordant case.

North America Inc), and no changes in their results were observed after the exclusion of cytologic cell blocks containing <100 cells. Approximately 31% of their histology specimens were small biopsies. Each of their surgical resection specimens was collected within 6 weeks of its corresponding cytology specimen, suggesting that the majority were resectable, early-stage tumors.[42] In addition, Bratton et al also reported concordance of PD-L1 positivity in 22 of 25 cytology-histology NSCLC specimen pairs, although they did not report the antibody used, the cutoff value used to determine positivity, or the collection method used for either cytology or histology specimens.[43] Finally, in an analysis of a subset of patients for whom both cytologic EBUS-FNA specimens and histologic biopsy or surgical

resection samples were available, Sakakibara et al observed poor correlation of PD-L1 expression (correlation coefficient, 0.19; $P = .49$) in 15 cases with 100 to 1000 tumor cells but good correlation (correlation coefficient, 0.68; $P = .0019$) in 18 cases with > 2000 tumor cells. Unfortunately, the number of those cases that were NSCLC was not reported, and the antibody used (EPR1161; Abcam, Cambridge, Massachusetts) was not among those used in conjunction with therapies that currently are approved by the FDA or in clinical trials, as outlined in the Blueprint comparison.[36] Overall, these reports corroborate the feasibility and concordance of PD-L1 expression between cytologic and histologic specimens as measured by IHC in the current study.

IHC Quantification of PD-L1 Expression: Cytology Specimens

The percentage of cytologic specimens with positive PD-L1 expression in the current study (40%) was observed to be higher than that of both histologic small biopsy specimens (23%) and histologic specimens overall (25%). Similarly, Bratton et al noted a slightly higher rate of positivity in ADC (60%) and SCC (60%) cytology specimens when compared with matched surgical resection specimens (40% and 53%, respectively), although they did not report the antibody used, the cutoff value used to determine positivity, or the collection method used for either cytology or histology specimens.[43] Although the difference in the current study did not reach statistical significance, it is an unexpected result, one that is at least partly attributable to tumor heterogeneity. In their study of PD-L1 in patients with NSCLC, McLaughlin et al observed heterogeneous expression by tumor cells among areas within sections of the same tumor block using 2 antibodies (E1L3N [Cell Signaling Technology, Danvers, Massachusetts] and SP142 [Ventana Medical Systems Inc, Tucson, Arizona]) that were different from the one used in the current study.[44] It is interesting to note that, in a subsequent study, researchers from the same laboratory observed a high interclass correlation coefficient for expression in NSCLC tumor cells between sections from different blocks using a single rabbit monoclonal antibody (SP142).[45] Despite differences with regard to within-block and between-block heterogeneity of PD-L1 expression, and the contribution made by the choice of antibody notwithstanding, the nature of FNA biopsy is likely to mitigate the effects of expression heterogeneity. Whereas a single core needle biopsy extracts tissue from a single site, an FNA biopsy is performed using a tissue-disruptive, back-and-forth motion. It is possible that greater areas have been sampled with dynamic FNA but not static core needle biopsies. Alternatively, higher levels of PD-L1 expression in cytology samples compared with histology samples may be attributable to tumor stage. In both studies of heterogeneity of PD-L1 expression, the majority of patients had early-stage disease. Although clinical staging information was not recorded in the current study, it is possible that the patients for whom PD-L1 expression was quantified using cytology specimens generally had a later stage of disease compared with those for whom PD-L1 expression was quantified using histology specimens. The possibility that increased PD-L1 expression correlates with a later stage of disease requires further investigation, and such a possibility would not account for the difference in PD-L1 expression noted between cytologic and small histologic biopsy specimens.

It is important to note that there was no difference with regard to the rate of PD-L1 positivity noted between ADC (29%) and SCC (18%). The lack of a difference in PD-L1 expression among tumors of different morphologic subtypes concurs with the results of a previous study that noted no significant difference in PD-L1 expression between ADC and SCC.[41]

Primary Versus Metastatic Sites

A modest number of matched primary and metastatic tumor specimens also were included in the current study, and only 2 pairs demonstrated discordance of PD-L1 expression. This contrasts with the results of Mansfield et al, who observed discordant PD-L1 expression using E1L3N in 10 of 73 matched primary lung and metastatic brain NSCLC specimens.[46] Mansfield et al claimed that the majority of paired lesions with discordant tumor cell expression of PD-L1 were obtained ≥ 6 months apart, although their data were not available. It is important to note that 1 of the 2 discordant pairs in the current study consisted of a primary lung tumor and its brain metastasis obtained 2.5 years apart and after chemotherapy and EBRT directed at both the lung and brain. To the best of our knowledge, heterogeneity in PD-L1 expression patterns among tumors of different types, as well as differences in tumor cell PD-L1 expression in immune privileged sites such as the central nervous system, have not been well characterized and may be a target of future investigation.

Reproducibility

Among specimens for which PD-L1 expression was requantified by separate pathologists, there were no cases for which the final interpretation changed, positive versus negative, even in cases with expression levels close to the 50% cutoff value. However, reproducibility of quantification was not perfect, with minor numerical discrepancies in 5 of 30 reviewed cases. The principal confounding factor was the presence of macrophages immunoreactive with the anti-PD-L1 antibody. In some cases, immunoreactive macrophages were similar in size to neoplastic cells, thereby complicating the distinction between the 2 on the IHC slide. In histology specimens, macrophages and ADC cells (micropapillary structures and single cells) coaggregated in some alveolar spaces. In cytology effusion specimens, quantification of PD-L1 expression was challenging when discohesive neoplastic cells were singly dispersed among macrophages. This challenge remained even after correlation with IHC to identify neoplastic cells, because the location of single neoplastic cells may vary from slide to slide. A PD-L1 antibody cocktail that differentially marks macrophages and neoplastic cells could improve the reproducibility and accuracy of the quantification of PD-L1 expression.

Among the limitations of the current study, the small number of matched primary surgical resection and small biopsy specimens is conspicuous yet not unexpected because patients with advanced disease are unlikely to undergo surgical resection in routine clinical practice. To address the paucity of matched surgical resection specimens, we evaluated a large number of consecutive and contemporaneous histology samples without selection bias to capture similarities and differences in staining among them. Rates of PD-L1 positivity across different sample types (cytologic and histologic) were similar. The results of the current study also addressed testing for the prediction of response to a single immunotherapeutic agent (pembrolizumab) using a single IHC marker (22C3 pharmDx), although there are other immunotherapeutic agents for NSCLC that are approved by the FDA or currently in clinical trials and, similar to pembrolizumab, each has an accompanying in vitro diagnostic test.

Quantification of PD-L1 expression on cytology and other small biopsy specimens of NSCLC is feasible using at least one of several commercially available IHC assays. Furthermore, quantification is comparable between surgical resection and small biopsy specimens, including in a small number of matched specimens, despite previous reports of tumor heterogeneity. This is important because cytology and small biopsy specimens often represent the only available tissue, especially in patients with advanced and unresectable disease. Further investigation is required to determine whether immunotherapy after PD-L1 quantification using cytology or other small biopsy specimens confers a survival benefit to patients with NSCLC.

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Adrian G. Sacher has received travel funding from AstraZeneca, Pfizer, and Genentech-Roche. Catherine A. Shu has served as a paid member of the advisory board of Genentech. Naiyer A. Rizvi is a paid member of the advisory boards for Merck, Roche, Bristol-Myers Squibb, Novartis, Pfizer, Lilly, and AbbVie and the scientific advisory board of Nilogen Oncosystems and is a co-founder and shareholder in Gritstone Oncology. Anjali Saqi holds a US patent (20150289856) on a cell block device and has consulted for Boston Scientific.

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